Chemistry & Biology 16

Supplemental Data

In Vivo Fluorescent Detection of Fe-S

Clusters Coordinated by Human GRX2

Kevin G. Hoff, Stephanie J. Culler, Peter Q. Nguyen, Ryan M. McGuire, Jonathan J. Silberg, and Christina D. Smolke

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Analytical gel filtration

A Superdex 75 column driven by an AKTA FPLC system (GE Healthcare) was used to separate monomeric and dimeric Venus-GRX2 immediately prior to spectral measurements and to evaluate the extent to which Venus-Gcn4 had been converted to monomer by enterokinase. All gel filtration experiments involving these proteins were performed using columns that had been equilibrated in PSG buffer. The apparent molecular weights of the eluted proteins were estimated using a standard curve generated with BSA (66 kDa), carbonic anhydrase (29 kDa), and cytochrome C (12.4 kDa).

Bacterial expression and protein purification

Rosetta2 cells harboring a pET vector for expressing the Venus-GRX2 and Venus-Gcn4 fusion proteins were grown in LB containing 10 μg/mL kanamycin at 37^{o}C, induced with 0.1 mM IPTG at $A_{600} \approx 1$, and grown for 18 h at 23°C. Cells expressing Venus-GRX2 and Venus-Gcn4 were lysed and purified using an NTA column as described above. Venus-Gcn4 was subsequently chromatographed using a Superdex 75 gel filtration column that had been equilibrated using PSG buffer (50 mM phosphate 7.0, 300 mM NaCl, and 2 mM GSH). Concentrations of purified Venus-GRX2 (ε_{280} = 29.4 $mM^{-1}cm^{-1}$) and Venus-Gcn4 (ε_{280} = 23.8 $mM^{-1}cm^{-1}$) were determined spectrophotometrically, and purified proteins were stored at –80°C.

Enzyme assays

The activities of lactate dehydrogenase and xanthine oxidase were determined in mock, NFS1 depleted, and ISCU depleted HEK293 cells. Lactate dehydrogenase activity was determined for 4 μg of total cellular lysate using a lactate dehydrogenase assay (Buffalo Research Service Center, Cat # E-107) as recommended by the manufacturer. Xanthine oxidase activity was determined for 24 μg of cellular lysate using the Amplex Red xanthine/xanthine oxidase assay kit (Invitrogen) according to the manufacturer's protocol. Fluorescence changes were determined using 560 nm excitation and 590 nm emission on a Tecan Saphire plate reader.

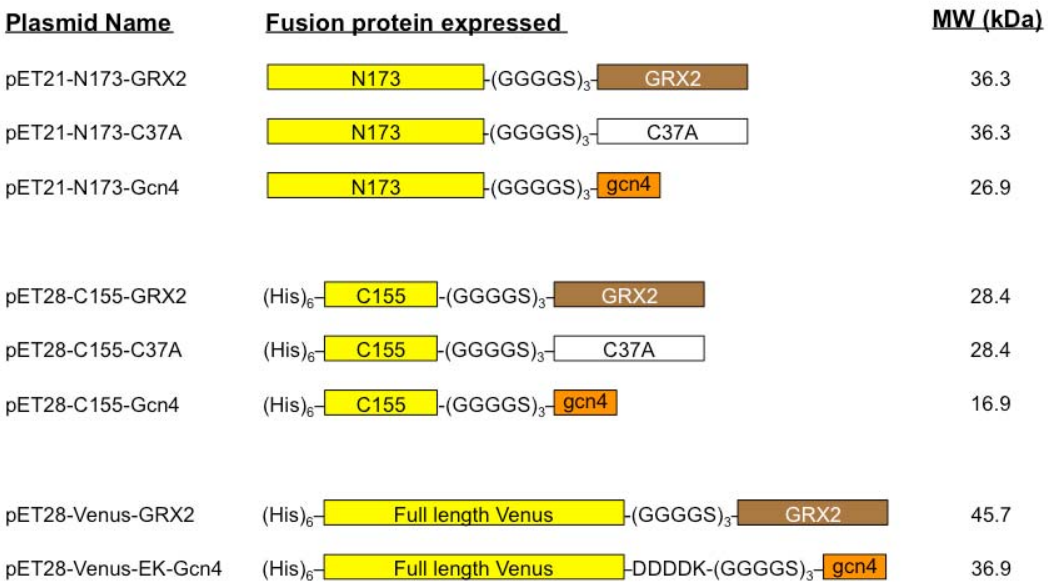

Figure S1. Bacterial vectors used for expressing fusion proteins
C155 fusion genes were cloned into pET28a using *bamHI* and *notI* to generate kanamycin-resistant vectors that express fusion proteins with N-terminal $(His)_6$ tags, whereas N173 fusion genes were cloned into pET21d using *bamHI* and *notI* to generate ampicillin-resistant plasmids that express fusion proteins that lack affinity tags. Genes encoding full-length Venus were also cloned into pET28a to allow for expression with a hexahistidine affinity tag.

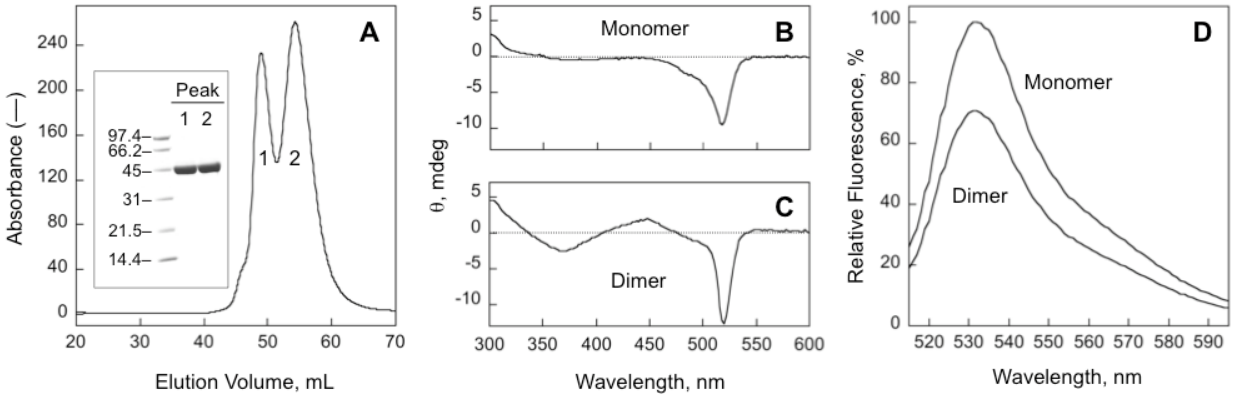

Figure S2. 2Fe2S clusters binding to Venus-GRX2 decreases fluorescence
(A) Elution profile of purified Venus-GRX2 chromatographed using a Superdex 75 column (solid line). A comparison of the peak elution volumes for Venus-GRX2 with standards of known molecular weights indicates that peak 1 exhibits an apparent molecular weight consistent with that of a dimer, whereas peak 2 displays an apparent molecular weight consistent with that of a monomer. *Inset*, SDS-PAGE analysis of 10 µg Venus-GRX2 from each elution peak.
Circular dichroism spectra of the (B) slow and (C) fast migrating Venus-GRX2 (10 µM each) reveal that the higher molecular weight form of the fusion protein has ellipticity minima (370 nm) and maxima (450 nm) similar to those observed in the spectrum of 2Fe2S-bound dimers of GRX2 (Lillig, et al. 2005).
(D) Fluorescence spectra of monomeric and dimeric Venus-GRX2 (10 µM each) indicate that the fluorescence of the 2Fe2S-cluster bound dimer is 30% lower than that of the monomer. All spectra are corrected for buffer absorbance and ellipticity.

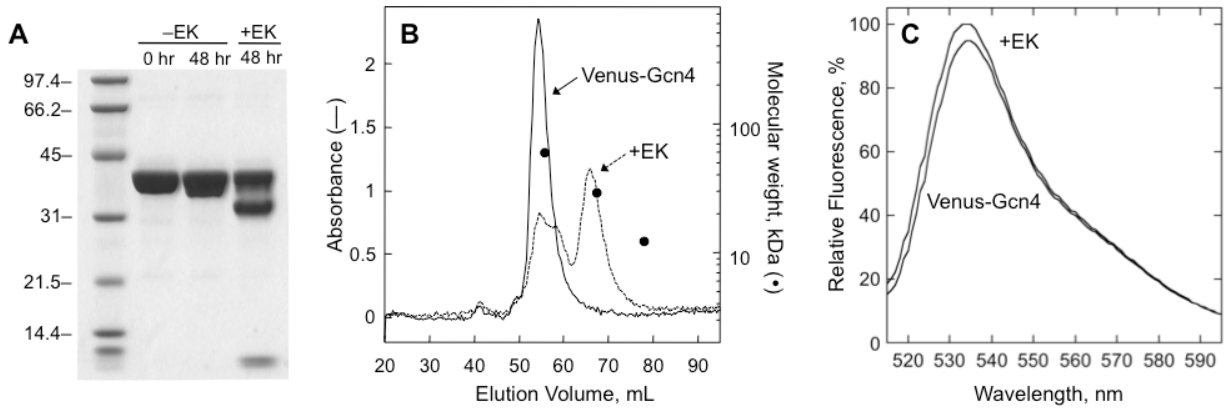

Figure S3. Effect of Venus proximity on fluorescence
(A) SDS-PAGE analysis of Venus-Gcn4 that was incubated for 48 hours in the presence and absence of enterokinase.
(B) The elution profile of enterokinase-treated Venus-Gcn4 (dashed line) was compared with untreated protein (solid line) using a Superdex 75 column. Protein standards (●) of known molecular weight are shown for reference.
(C) Enterokinase treatment increases the fluorescence of Venus-Gcn4. Untreated, dimeric Venus-Gcn4 exhibits 5% lower fluorescence than the proteolyzed Venus-Gcn4, which contains a 1:1 mixture of monomer and dimer. This indicates that Venus molecules brought into close proximity by dimerizing Gcn4 molecules exhibit ~10% lower fluorescence than monomeric Venus. Spectra are corrected for buffer fluorescence.

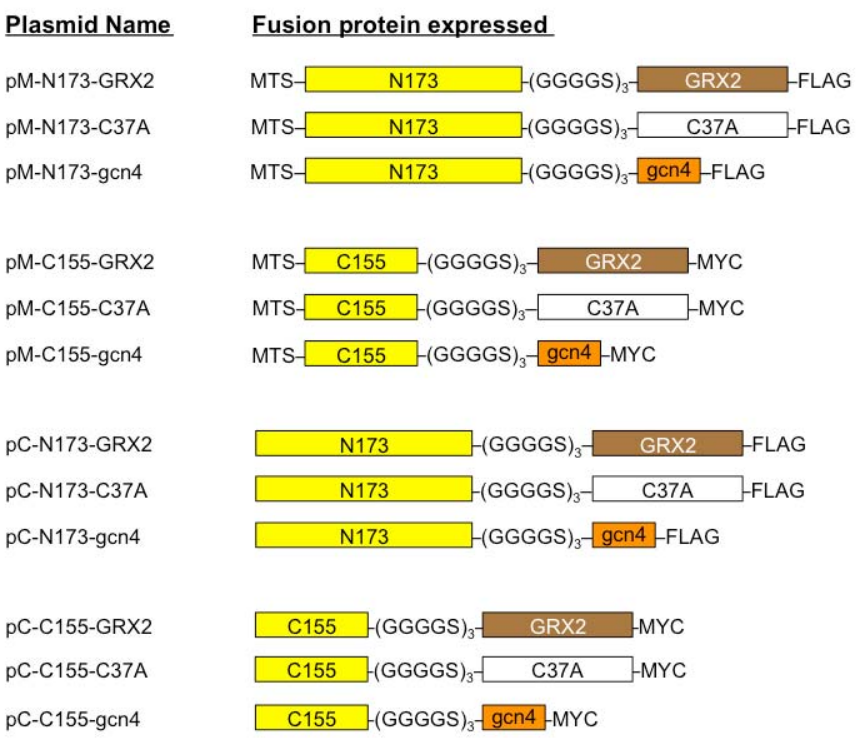

Figure S4. Mammalian vectors used for expressing fusion proteins
All mammalian vectors that express N173 were designed to generate fusion proteins with C-terminal FLAG tags (DYKDDDDK), whereas all vectors that express C155 were designed to generate proteins with C-terminal Myc tags (EQKLISEEDL). Cytosolic constructs were generating by cloning gene fusions into pcDNA5/frt using *bamHI* and *notI*, and mitochondrial constructs were generated from these vectors by cloning the cytochrome c oxidase subunit 8 mitochondrial targeting sequence in front of the gene fusions using *ecoRI* and *bamHI*.

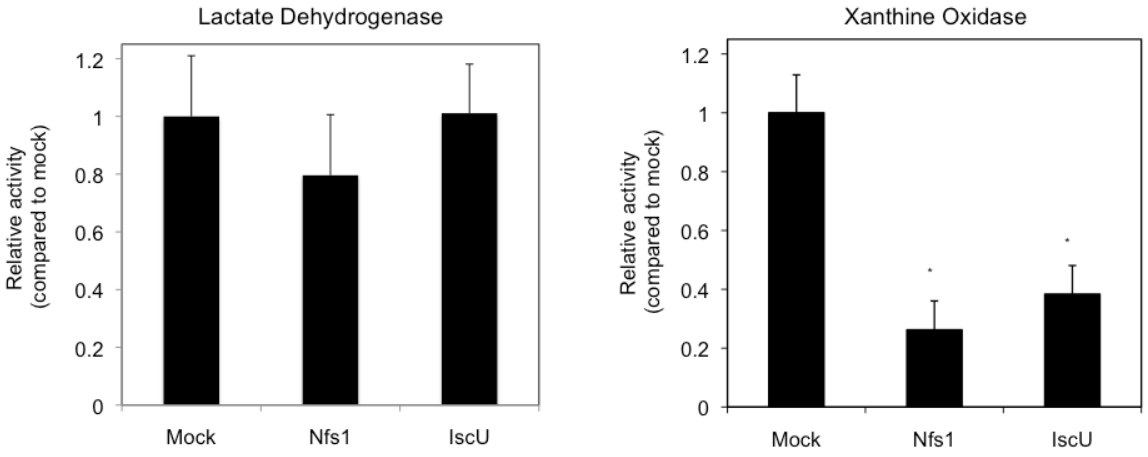

Figure S5. ISCU and NFS1 depletion decrease the activity of xanthine oxidase, an Fe-S dependent enzyme

The catalytic activity of xanthine oxidase was compared to that of an enzyme that does not require an Fe-S cluster to function (lactate dehydrogenase) in cells transfected with a siRNA duplex targeted to *ISCU*, *NFS1,* and a mock siRNA. Shown are the means and +/- S.D. (n=3). *, $p<0.01$ (T-test).